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13. ABSTRACT

In experimental frostbite, more severe injury is seen in slowly thawed tissues than in rapidly thawed tissues frozen for the same length of time. The extent of healing which follows a given exposure time depends upon the manner of thawing, the area sectioned, and the duration of freezing. The most striking histologic differences between slow- and rapit-thawed tissues occur in the endothelium, internal elastic lamina and media. Following slow thawing, the endothelial cells are almost completely shed into the lumen. The internal elastic lamina is disrupted and, as early as one hour after exposure, exhibits areas of decreased stainability. The medial muscle cells are distorted and twisted. The media progressively loses its normal architecture so that the end picture is that of liquefaction necrosis. Following rapid thawing, the endothelial cells remain attached to the intima. Three days after exposure, these cells are hyperchromatic and actively proliferating and the media contains numerous hyperchromatic cells. By six days, intimal proliferation is readily apparent and the internal elastic lamina of rapidly thawed arteries remains intact and demonstrates only occasional areas of loss of stainability. The medial muscle cells are much less distorted and twisted. In comparison to slow-thawed tissues, the initial injury appears less severe, and repair is initiated earlier and is more complete. This is not without complications, however, in that reactive intimal proliferation appears to progress to partial or total occlusion of the involved vessels.

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Vascular Injury Due to Cold

Effects of Rapid Rewarming

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In experimentally induced frostbite, more severe injury is seen in slowly thawed tissues than in rapidly thawed tissues. Histologic changes described here show vascular reactions which correlate with tissue survival studies. The experimental procedure was a standard injury to the rabbit hind limb, followed by either rapid rewarming (hot water) or spontaneous thawing at room temperature. Following slow thawing, endothelial cells are almost completely shed into the lumen, the internal elastic lamina is disrupted, and medial cells are distorted and twisted and become necrotic. Following rapid thawing, endothelial cells remain attached to the intima and are actively proliferating after three days, the internal elastic lamina remains intact, and medial cells are less distorted. The initial injury of rapidly thawed tissue appears less severe; repair is initiated earlier and is more complete. Surviving vessels present varying degrees of irreversible endarteritis obliterans.

THE BEST treatment for frostbite is now believed to be rapid rewarming of the affected part in hot water (42 C). It has been shown experimentally in the laboratory that of the many treatments tried, this one results in the most tissue survival. Much of the evidence is summarized in review articles by Lewis[1] and Lapp and Juergens.[2] Clinical observations seem to agree with the laboratory findings.[3-5] How rapid rewarming produces a beneficial effect is not really known, although one factor may be the production of maximal blood flow in the injured area brought about by the dilating effect of heat on the blood vessels at the time of thawing.

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There is little doubt that vascular alterations play a role in the development of tissue necrosis following freezing injury, be it by direct injury to the vascular tissue or from vascular reaction to abnormal metabolites released by other damaged cells. The following is a report on a histological study of vascular lesions in the rabbit foot exposed to a standard freezing injury, and on the effect of rapid and slow rewarming on the recovery from the injury. The histologic findings support the clinical observation that rapidly rewarmed tissue has a better chance of survival than slowly rewarmed tissue.

Materials and Methods

Adult albino rabbits weighing more than 2,500 gm were used. Under pentobarbital sodium anesthesia, these animals were subjected to a standard freezing injury, the details of which have previously been published.[6] In brief, the hind limb of each animal was exposed to a freezing mixture kept at a temperature of −15 C. The duration of freezing was indicated by a thermocouple located deep within the interosseous space between the second and third metatarsal bones (Fig 1). The animals were divided into four groups as indicated in Fig 2. In groups 1 and 4, the frozen foot was allowed to thaw in air at room temperature, while in groups 2 and 3 the foot was immersed in a water bath at 42 C immediately upon removal from the cold bath and kept in the bath until the foot temperature reached the level of the animal's rectal temperature. All animals were given aqueous penicillin G benzathine and were returned to individual cages. Two rabbits each of groups 1 and 2 were killed at the following intervals after exposure: 15 and 30 minutes; 1, 2, 3, 4, 6, and 12 hours; and 1, 2, 3, 4, 5, 6, 9, 12, 16, 18, 20, 24, 30, and 40 days. Eleven and 18 animals of groups 3 and 4, respectively, were included. Immediately after death, a 2-cm tissue block was dissected from the metatarsal area of the plantar surface of the foot and fixed in 10% neutral formaldehyde solution (formalin). Control sections came from the opposite foot and from randomly selected stock animals.

Sections of the fixed tissues were stained with hematoxylin-eosin, Mallory's phosphotungstic acid-hematoxylin, Masson's, Verhoeff's stain for elastic tissue, and the PAS technique.

Results

Gross Observations.—Upon removal from the freezing mixture, the foot was observed to be frozen up to the line of immersion (the tarsal-metatarsal junction). The involved portion of the extremity appeared waxy and ashen and was frozen solid.

In those animals allowed to thaw slowly (groups 1 and 4), the foot temperature reached a level of −2 C quickly, and then remained near this temperature for 20 to 30 minutes. The temperature then rose rapidly to body temperature level (Fig 2). During this period, the skin gradually developed a faint pink color. One hour after exposure, the foot was bright pink and had become quite turgid. Within two hours, the color became bright red, and the skin was covered by a clear transudate. Due to massive ede-

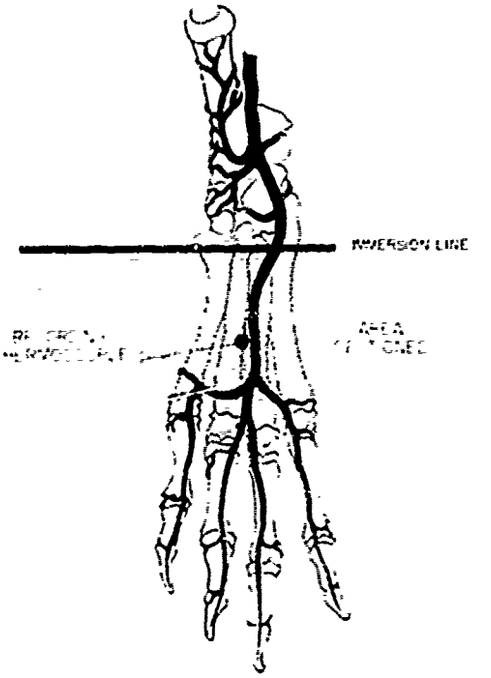
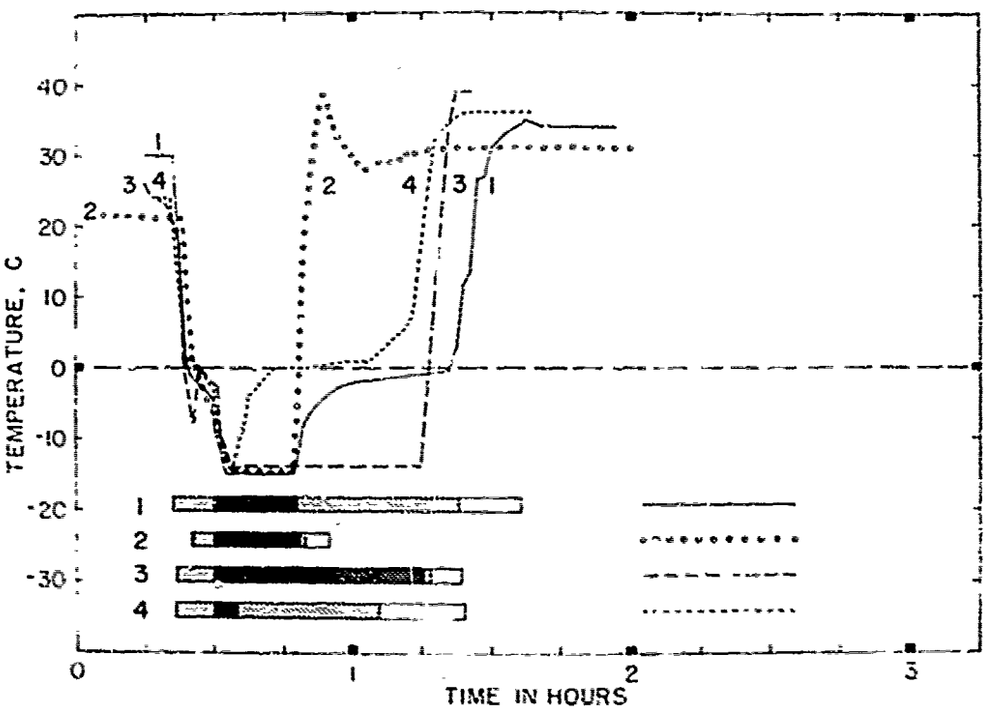


Fig 1.—Diagram of rabbit foot showing plantar arterial arch, site of deep thermocouple, and immersion line.

Fig 2.—Temperature curves of deep foot tissue, one representative of each of the four experimental groups. Feet of animals in groups 1 and 2 were kept in cold bath for 18 minutes after freezing occurred; then group 1 feet were thawed slowly (air) and group 2 feet rapidly (hot water). Group 3 feet were kept frozen 45 minutes before being rapidly thawed. Group 4 feet were kept frozen only until deep tissue temperature reached bath temperature, then thawed slowly. Time frozen in the bath is represented by **cross hatches**; time in bath before freezing occurred, by **hatch lines slanting up left to right**; time out of bath still frozen, by **hatches slanting down left to right**; and time thawed but below maximum rewarmed temperature, by **open blocks**.



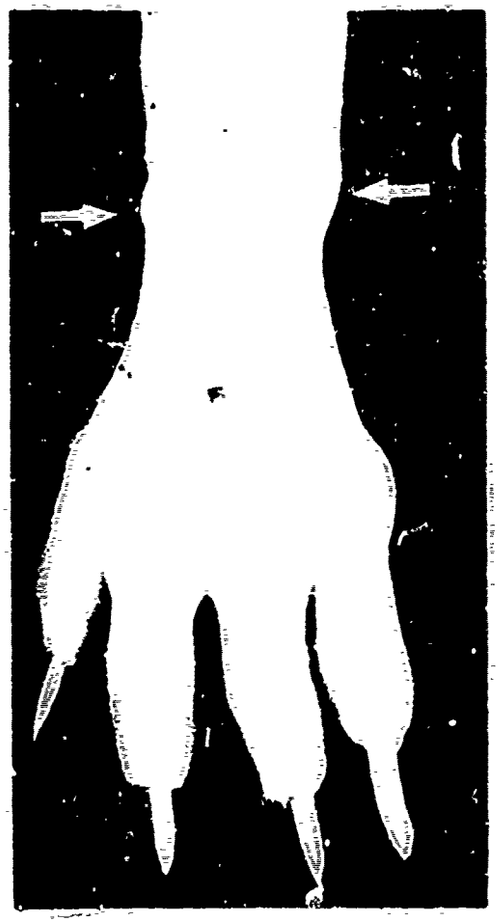


Fig 3.—Gross appearance of rabbit foot two to four hours after completion of slow thawing (group 1). The foot is markedly swollen due to edema. Arrows indicate level of immersion line.

ma, the foot assumed the appearance of a rubber glove distended with water (Fig 3). During the next few hours, the deeper tissues gradually assumed a blue tinge. On the following day, the skin was dusky red and irregularly covered by a grey transudate. The foot was wet and cool. The edematous condition persisted for three to four days, after which time the fluid decreased in amount. The skin then became wrinkled and loose, then dry, hard, and black. The line of demarcation between black, dry, mummified tissue and pink, soft, viable tissue became sharp after five to seven days (Fig 4).

This sequence of events was noticeably altered in extremities that were rapidly rewarmed (groups 2 and 3). Thawing was

Fig 4.—Gross appearance of rabbit foot five to seven days after slow thawing (group 1). The immersion line (arrow) is distinctly delimited by the gangrenous foot and digits.

accomplished in four to six minutes. The skin became blue while the foot was still in the warm bath and remained blue for as long as three days. Edema and cyanosis were much more marked after two hours than at the same time interval in slowly thawed limbs. The feet were warm, moist, and swollen after 24 hours. After three days, the phalanges were in many cases dusky gray and gangrenous, while the dorsal foot pad remained warm and blue. Five days after exposure, a dark, dried eschar could be peeled from underlying viable pink tissue. The foot stub remained larger than normal for over 40 days, and the skin was red, thin, and shiny.

Evaluation of tissue survival showed results similar to those previously reported[6]: poor in groups 1 and 4, with no survival below the immersion line; good in group 2 with survival below the immersion line in all animals, sometimes including phalangeal tissue; and half and half good and poor in group 3.

Microscopic Findings.—*Slowly Thawed Tissues.*—During the first hours after termination of freezing, one of the most striking phenomena is the progressive shedding of

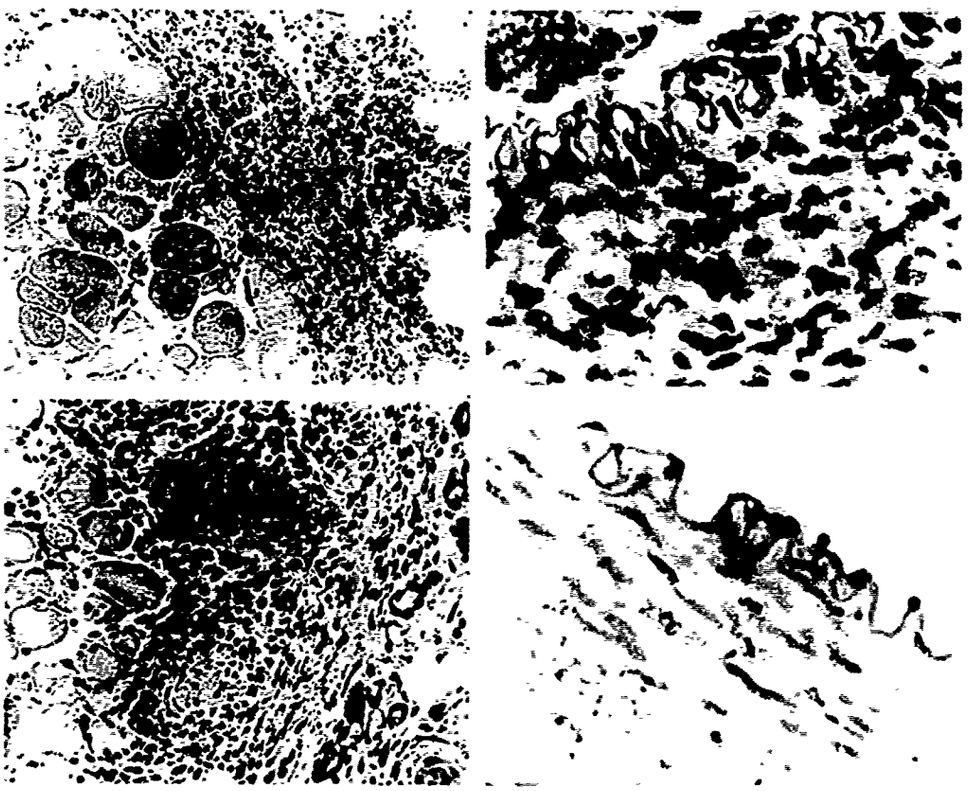


Fig 5.—**Top left,** Deep plantar muscle and connective tissue three days after slow thawing (group 1). Note the muscle necrosis, absence of proliferaton of granulation tissue, and presence of edema and hemorrhage (hematoxylin-eosin, ×204). **Top right,** Deep planar artery from midfoot 12 hours after slow thawing (group 1). Many endothelial cells are absent. The media contains necrotic muscle cells, granulocytes, and erythrocytes (hematoxylin-eosin, ×816). **Bottom left,** Same as **top left** but following rapid thawing (group 2). Note proliferation of granulation tissue and preservation of sarcolemma cells (hematoxylin-eosin, ×204). **Bottom right,** Same tissue and time interval as **top right,** but following rapid thawing (group 2). Endothelial cells are for the most part still present. The media contains curled muscle nuclei and focal areas of decreased ground substance hematoxylin-eosin, ×816).

endothelial cells into the lumina of the blood vessels. The sequence of endothelial morphology appears to be pyknosis followed by loss of cohesion with the adjacent intima (Fig 5, *top right*). During this same time, the internal elastic lamina becomes irregularly split and fragmented. Increasing amounts of elastic tissue lose their affinity for eosin and for Verhoeff's stain for elastic tissue.

The media likewise exhibits striking changes. Curling and twisting of muscle nuclei is followed by progressive pyknosis, karyolysis, and karyorrhexis. The adjacent ground substance becomes increasingly pale and vacuolated. By four hours after cessation of cold exposure, the arteries and veins contain segmental areas of liquefaction necrosis. Beginning about three to four hours after termination of cold exposure, the walls of the vascular system and the adjacent supporting tissues contain increasing numbers of both erythrocytes and granulocytes. By 12 hours postexposure (Fig 5, *top right*), exudation is associated with edema, hemorrhage, and necrosis throughout the walls of the vascular system, which progress in intensity (Fig 6, *top*). Throughout the initial four-hour period, the adjacent tissues exhibit increasing distortion and vacuolation of skeletal muscle bundles accompanied by capillary congestion. After 24 hours these tissues contain areas of suppuration.

Proliferation of granulation tissue is con-

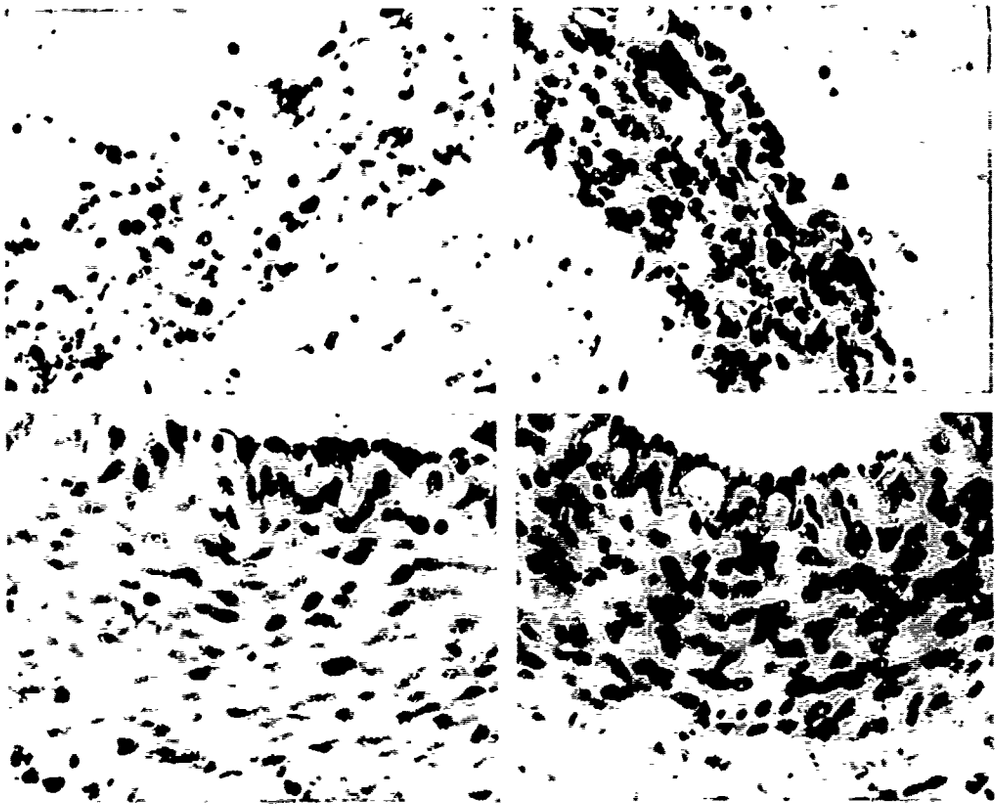


Fig 6.—Top left, Deep plantar artery from midfoot three days after slow thawing (group 1). Note complete necrosis of intima and media (hematoxylin-eosin, ×408). Top right, Same as top left but from group 4. Note the focal area of intimal proliferation. The media contains cellular debris, hemorrhage, and granulocytes (hematoxylin-eosin, ×408). Bottom left, Same as top left, but rapidly thawed (group 3) (hematoxylin-eosin, ×816). Bottom right, Same as bottom left but group 2. The endothelial cells are actively proliferating as are certain cells in the media (×816).

spicuously absent except for small focal areas surrounding some of the more superficial arterioles and venules. Vessels in the latter, superficial location are in general less damaged than the deeper vessels of the plantar arch. Mural necrosis of these deeper vessels (Fig 6, *top*) is associated, at three days, with necrosis of the adjacent skeletal muscle and hemorrhage and edema of the surrounding connective tissue (Fig 5, *top left*). By six days, necrosis and suppuration have progressed to gangrene of the entire foot up to the immersion line.

Rapidly Thawed Tissues.—The endothelial cells, for the most part, remain attached to the underlying intima (Fig 5, *bottom right*), but a few are desquamated, particularly from focal areas in which the intima is elevated by fibrin and erythrocytes. The internal elastic lamina remains intact, and there is much less curling and twisting of medial muscle nuclei. The supporting tissue become edematous immediately after thawing as opposed to the 30-minute delay that occurs in slowly thawed tissues. Likewise, rapidly thawed tissues contain capillary ring hemorrhages as early as 15 minutes after thawing, but these hemorrhages are rarely seen in slowly thawed sections before one hour. After one hour, comparison between rapidly and slowly thawed tissues reveals not only that the latter are more severely damaged, but that under these experimental conditions the equivalent reactions occur about 6 to 12 hours later in slowly thawed tissues than in rapidly thawed tissues. This comparison is summarized in the Table.

In rapidly thawed tissues, the absence of endothelial shedding is no less striking than is the presence of endothelial proliferation.

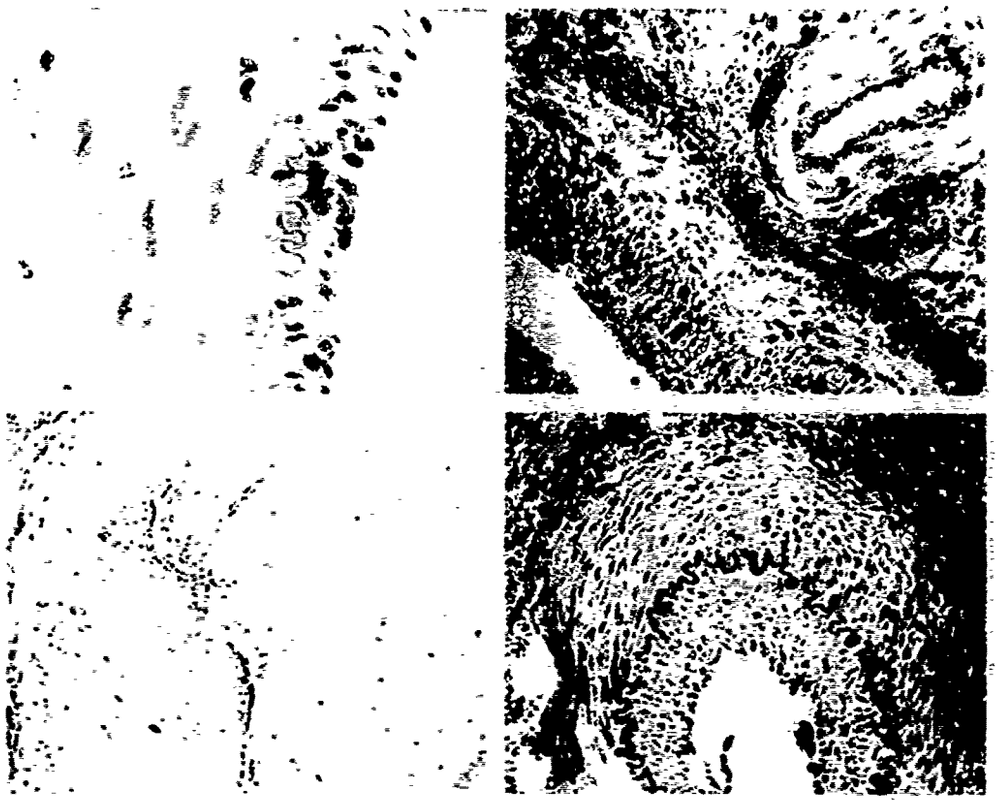


Fig 7.—**Top left.** Control deep plantar artery with intimal proliferation. This is an uncommon finding in controls (×816). **Top right.** Superficial artery and vein 27 days after rapid thawing. Note the absence of intimal proliferation in the artery, and the presence of marked intimal proliferation in the vein (Verhoeff's stain, ×204). **Bottom left.** Deep plantar artery 60 days after rapid thawing. Note the massive intimal proliferation (hematoxylin-eosin, ×204). **Bottom right.** Deep artery 27 days after rapid thawing. Note the marked intimal proliferation (Verhoeff's stain, ×204).

By three days, the endothelial cells from group 2 arteries (Fig 6, *bottom right*) are hyperchromatic and appear to be actively proliferating. At the same time interval, this phenomenon has progressed even further in group 3 arteries (Fig 6, *bottom left*), as is manifested by intimal thickening. In a given case, however, this degree of intimal thickening cannot be distinguished from the naturally occurring endemic intimal proliferation that one sees in a few rabbits (Fig. 7, *top left*). By three to four weeks (Fig 7, *top* and *bottom right*), however, intimal proliferation has progressed well beyond even the most striking example of the naturally occurring disease. By 60 days, many of the arteries of the plantar arch present the picture illustrated in Fig 7, *bottom left*.

Under these experimental conditions there are two other interesting proliferative phenomena that occur after rapid rewarming. One of these occurs in the media of arteries and veins and is manifested by hyperchromatism and pleomorphism of sarcolemmic and fibroblastic elements (Fig 6, *bottom*). In the case of the arteries, these cytologic changes do not indicate proliferation since they do not terminate, at least in 80 days, in medial fibrosis or thickening. These morphologic alterations in cells of the media may be related to redisposition of amorphous ground substance. Decrease of ground substance is apparent after three days and is particularly prominent about 14 days after termination of exposure (Fig 8, *top* and *bottom right*). Areas of decreased or absent ground substance take the form of distinct bands that gradually decrease in width as repair occurs. The cytoplasm of the medial cells increases in density, making the cellu-

Fig 8.—**Top left,** Deep plantar vein 21 days after rapid thawing. The wall has been replaced by cellular connective tissue (hematoxylin-eosin, ×204). **Top right,** Deep plantar artery 14 days after rapid thawing. Note the peripheral "clear" area of media with decreased or absent ground substance (hematoxylin-eosin, ×204). **Bottom left,** Deep plantar artery 27 days after rapid thawing. Note the intermittently broken internal elastic lamina (Verhoeff's stain, ×204). **Bottom right,** Same as **top right.** Note the focal areas of decreased ground substance in the media (hematoxylin-eosin, ×204).

lar outlines unusually distinct. As this process continues, increased density also gradually appears between the cells so that the cell outlines once again appear indistinct. This sequence suggests actual secretion and deposition of ground substance by medial cells. At any one time all phases of this reparative phenomenon are apparent in a given artery. In the case of veins, however, medial proliferative phenomena lead to replacement of the media by cellular fibrous tissue (Fig 8, *top left*). There appears to be no increase in elastic tissue in either arteries or veins. Conversely, the veins appear to lose elastic tissue, but we are unable to say whether this also occurs in the arteries.

Proliferative phenomena are also readily apparent in the surrounding skeletal muscle and connective tissue. Three days after termination of exposure, proliferation of granulation tissue is well under way and associated with considerable sarcolemmic activity (Fig 5, *bottom left*).

It is of importance to recognize that all of these processes may and often do occur in a segmental fashion. This segmental distribution is particularly likely to occur the nearer sections are taken to the immersion line. Any given section may show focal or segmental reactions. We have seen both intimal proliferation and medial necrosis limited to one side of an artery or vein. An example of focal loss of the internal elastic lamina is illustrated in Fig 8, *bottom left*.

Comment

It was shown in similar earlier experiments[6] that rapid rewarming had a

Comparison of Slowly and Rapidly Thawed Tissues

	Slowly Thawed	Rapidly Thawed
Edema	30 min	immediate
Capillary hemorrhages	1 hr	15 min
Desquamation of endothelial cells	Uniformly present	Almost absent
Fragmentation of internal elastic lamina	Uniformly present	Almost absent
Medial degeneration and necrosis	Marked	Minimal to moderate
Exudation	5 hr	4 hr
Beginning proliferation of fibroblasts	72 hr	24 to 36 hr
Endothelial regeneration	Almost completely absent	Well established at 72 hr
Medial proliferation	Almost completely absent	Well established at 72 hr
Capillary budding	Scattered superficially at 4 days	Well established at 72 hr
Gangrene	Entire foot	Digits only

beneficial effect per se on tissue survival, with rapidly thawed feet (group 3) showing better survival than feet which were slowly thawed (group 1) even though the rapidly thawed feet were actually in a frozen state twice as long as the slowly thawed. The poor tissue survival in the animals of group 4 is consistent with the previous observations.

While it has been established that rapid thawing increases tissue survival after freezing injury,[1-3] the reason for the effect is not known. There is apparently some effect besides the simple reduction of the time frozen. Alteration of ice crystal size is not a likely explanation since, insofar as speed of temperature change affects types of ice crystal formation, most mammalian freezing injuries are believed to fall under the category of slow freezing and thawing.[2-6] Other unknown factors also affect the histopathological changes that follow freezing injury; there is apparently a species tissue-dependent gradient of susceptibility to various types of injury and to different degrees of the same injury.[1,7-11] For example, Lewis[1] observed early degenerative changes in striated muscle. This was not present in the striated muscle of this series, but was most striking in the media of arteries and veins (*Fig 5, bottom right*).

Opinions concerning the pathogenic basis of local cold injury have been divided between investigators who have emphasized damage to the supporting tissues[1,9,12,13] and those who stress primary circulatory dysfunction.[1,7,14-17] Probably both factors are involved, their contribution depending on the nature of the injury, tissue-specific gradients of susceptibility, and experimental or environmental conditions. These experiments have shown primary damage in vascular tissue, which can be lessened by rapid rewarming. But, even with this beneficial treatment, signs of injury are still present. The concept of tissue injury includes both the initial damage and the response to the damage. Response includes repair. In the blood vessels examined, both primary damage and inflammatory and reparative responses to the damage have been observed. Other investigators have also noted loss of endothelium following freezing injury,[15,18-21] and later endothelial proliferation.[11,13-15,19-21] Subendothelial proliferation has also been observed in large vessels frozen directly by cryotherapeutic techniques.[22,23]

The study has shown that rapid rewarming has an accelerating effect on the histological sequence of changes which follow freezing injury. A corresponding enhancing effect has been seen by some in the pathophysiologic events which follow freezing injury, such as edema formation and rate of metabolism.[3,7,18]

Although there is less gross tissue loss after rapid rewarming, it is possible that the chronic sequellae could be as incapacitating as a more extensive loss of tissue. Chronic skin, nerve, and circulatory alterations are frequent clinical manifestations of frostbite injuries.[14,24] The proliferative intimal changes seen in the major vessels in these experiments, with sometimes almost complete occlusion of the lumen, could certainly lead to chronic vascular insufficiency.

Mention should be made of the control findings. The occurrence of minimal endarteritis obliterans in supposedly normal rabbit arteries would unquestionably complicate investigations concerned with studying minimal degrees of intimal proliferation. A related drawback to the use of mongrel dogs

for the study of arteriosclerosis has been recorded,[25] and similar changes occur in the cat.[26] However, the naturally occurring endarteritis obliterans we have observed is not as severe as that observed in rapidly thawed vessels 14 days after exposure and never approaches the massive changes seen after 60 days.

Conclusions

Gross and microscopic findings in slowly and rapidly thawed tissues, under this set of experimental conditions, indicate that rapid thawing, in comparison to slow thawing, leads to (*a*) an accelerated inflammatory reaction, (*b*) less severe vascular and supporting tissue injury, (*c*) earlier healing, and (*d*) decreased permanent tissue loss.

Histologic reactions to cold injury are evident in vascular and supporting tissues immediately after thawing. Surviving rapidly thawed vessels present varying degrees of irreversible endarteritis obliterans. Control rabbits show a mild, naturally occurring, endarteritis obliterans.

This communication represents research task NM 41 02 00 from the Bureau of Medicine and Surgery, Navy Department.

The experiments reported herein were conducted according to the principles set forth in the *Guide for Laboratory Animal Facilities and Care* prepared by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

Nonproprietary and Trade Names of Drug

Penicillin G benzathine—*Bicillin, Permapen, Bicillin L-A.*

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